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Isolation and Growth of Adult Human Epidermal Keratinocytes in Cell Culture

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Human epidermal keratinocytes may be isolated in high yield from 0.1 mm keratotome sections of adult skin by short-term trypsin release. When plated on a collagen-coated plastic surface or on a collagen gel, keratinocytes attach with high efficiencies (>70%) and form confluent, stratified cultures. Cell populations of predominantly basal cells produce proliferative primary cell cultures while populations of basal cells and malpighian cells result in nonproliferative primary cultures. Both nonproliferative and proliferative primary cultures may be subcultured on collagen gels following dispersion by trypsin and EDTA.

Methotrexate strongly inhibits proliferative keratinocytes at low concentrations (0.1 μg/ml) but has no cytotoxic effect on nonproliferative cells. L-serine and dexamethasone increase the multiplication rate of both primary and subcultured human keratinocytes.

The ability to isolate and to grow human epidermal keratinocytes from both normal and diseased human skin in sufficient quantities for biochemical and genetic studies has been a long-range goal of many investigators. Although keratinocytes may be obtained from postembryonic skin by proteolytic release with trypsin [1–5], a limited growth period in cell culture is usually observed, and long-term growth of adult cells has, in most cases, been difficult to attain. Recently, Rheinwald and Green have described the growth of keratinocytes from foreskin when the cells are plated and grown in the presence of lethally irradiated mouse 3T3 cells. [1], and Freeman *et al* have reported better growth when minces of foreskin are serially transferred on killed pig skin [6].

When grown *in vitro*, keratinocytes retain many of the characteristics associated with the maturation of keratinocytes *in vivo*. These properties include the synthesis of proteins with histochemical reactions typical of keratinized cells [7], the formation of comified envelopes [8], and stratification [3]. The biochemistry, histology, and morphology of the maturation of human keratinocytes *in vitro* has been summarized recently [9].

In the present study we report a procedure to isolate and to cultivate human keratinocytes from adult skin in high yield, in the absence of feeder cells, and with high-plating efficiencies. The growth profiles of primary and subcultured cells are described, and some of the conditions and factors that influence the growth of adult keratinocytes are presented.

MATERIALS AND METHODS

Chemicals and Growth Media

Minimal Essential Media (MEM) and McCoy's 5A Medium were purchased in powder form from Microbiological Associates and Pacific Biologicals. Calf and fetal calf sera were obtained from Irvine Scientific Sales Co., Inc. Methotrexate and Trypsin (1–250) were products of ICN Pharmaceuticals, Inc. Dexamethasone was obtained from Sigma Chemical Co., and 35-mm plastic Petri dishes from Lux Scientific Corporation.

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Abbreviation: MEM, Minimal Essential Media

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Preparation of Collagen Surfaces

Acid soluble collagen is extracted and purified from adult rabbit skin as described previously [10]. Three types of culture surfaces are prepared on 35-mm plastic Petri dishes: (a) collagen-coated, (b) thin gel, and (c) 2-mm collagen gel.

(a) Collagen-coated dish. 1.0 ml of acid soluble collagen (dry wt 2.5 mg/ml) is placed in a 35 × 10-mm plastic Petri dish for 10 min. Collagen in excess of the amount required to coat the bottom of the dish is removed by aspiration, and the dishes are rinsed with sterile 0.15 M NaCl containing 400 units/ml penicillin and 200 ng/ml streptomycin, followed by a second wash for 30 min in sterile 0.15 M NaCl. The dishes are conditioned at 37 °C with MEM containing 10% calf serum for at least 1 hr before use, or are stored at 37 °C in complete growth medium if not used immediately.

(b) Thin-gel dish. 1.0 ml of acid soluble collagen is placed in a 35-mm plastic Petri dish, and the excess collagen is removed by aspiration. The dishes are placed in a dessicator and exposed to vapors of concentrated ammonia for 30 min. The dishes are removed and allowed to stand in air for 30 min to allow diffusion of excess ammonia from the gel. The gels are washed with 0.15 M NaCl as described above; 2 additional rinses of NaCl are used to assure complete removal of ammonia. The gels are conditioned and stored as described for a collagen-coated dish.

(c) 2-mm collagen gel dish. 1 ml of acid soluble collagen is placed in a Petri dish and the dishes are exposed to ammonia vapor to form a 2-mm gel, as described for a thin gel. The dishes are washed and conditioned as described for a thin-gel dish.

Preparation of Skin

Normal adult human trunk skin is obtained after autopsy and excess facial skin after meloplasty. The underlying fat is trimmed, and the tissue is rinsed 1 × in Hanks' balanced salt solution containing 400 units/ml penicillin, 200 μg/ml streptomycin, and 75 units/ml mycostatin for approximately 1 h. The epidermis and papillary dermis are removed with a Castroviejo keratotome set at 0.1 mm.

Isolation of Keratinocytes and Preparation of Primary Cultures

Three populations of keratinocytes are isolated from keratotome slices: (a) nonproliferative, (b) slowly proliferative, and (c) rapidly proliferative.

(a) Nonproliferative primary cultures. 0.1-mm keratotome slices (2 × 2 cm) are incubated at 37 °C in 0.3% trypsin in GNK (0.15 M NaCl, 0.04% KC1 and 0.1% glucose, pH adjusted to 7.6 with $NaHCO_3$) for 40–50 min. The slices are washed 3 times in 0.15 M NaCl, and transferred to complete growth medium (MEM, 10% calf serum heat-inactivated for 30 min at 56 °C). The epidermis and dermis are separated in complete growth medium, and basal and malpighian cells are released into the medium by gentle agitation of both the dermal and the epidermal compartments. Following agitation the dermis and stratum corneum are discarded, and the dispersed basal and malpighian cells remaining in the medium are counted in a hemocytometer. The appropriate dilutions are made with complete medium, and 1.0 ml of the cell suspension is plated immediately on a 2-mm collagen dish and incubated in an atmosphere of 95% air, 5% CO_2 at 37 °C. The cells are allowed to attach for 18 hours, the original plating medium is removed, and the cells are then fed at 3 day intervals with MEM containing 10% heat-inactivated calf serum.

(b) Slowly-proliferative primary cultures. To obtain slowly-proliferative cultures, basal and malpighian cells are obtained as described for